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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :
MECHTHILD RIEPING, ET AL. : EXAMINER: STEADMAN
SERIAL NO: 10/076,416 :
FILED: FEBRUARY 19, 2002 : GROUP ART UNIT: 1656
FOR: PROCESS FOR THE :
FERMENTATIVE PREPARATION OF L-
AMINO ACIDS USING
ENTEROBACTERIACEAE STRAINS
WITH ATTENUATED POXB GENES

APPEAL BRIEF

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

In accordance with 35 U.S.C. § 134, that the claims of the present application have been twice rejected, this brief is submitted in response to the rejections dated November 20, 2009 ("Action").

REAL PARTY OF INTEREST

The real party of interest is Evonik Degussa, GMBH of Germany.

RELATED APPEALS AND INTERFERENCES

To the best of Appellants' knowledge, there are no other appeals or interferences which will directly affect or be directly affected by, or have a bearing on, the Board's decision in this appeal.

STATUS OF CLAIMS

Claims 23, 25-28, 33 and 35-42 are active.

Claims 23, 25-28, 33 and 39-42 are rejected.

Claims 35-38 are objected.

Claims 23, 25-28, 33 and 39-42 are appealed.

The appealed claims are presented in Appendix I.

STATUS OF AMENDMENTS

No outstanding amendments are present in this case as all amendments are to be entered for purposes of an Appeal (see Advisory Action of August 9, 2010).

SUMMARY OF CLAIMED SUBJECT MATTER

The invention claimed in the pending, rejected and appealed independent claim 23 is:

A process for fermentatively preparing an L-amino acid selected from the group consisting of L-threonine, L-valine, and L-lysine, comprising *page 1, lines 7-11; page 2, lines 13-25; page 3, lines 6-19*

fermenting a modified microorganism of the genus Escherichia which already produces L-amino acids before being modified for a time and under conditions suitable for the production of the L-amino acid; *page 1, lines 7-11; page 2, lines 13-25; page 3, lines 6-19*

concentrating the L-amino acid in the medium or in the Escherichia cells; *page 1, lines 7-11; page 2, lines 13-25; page 3, lines 6-19*

determining the concentration of the L-amino acids produced; and *Example 1, pages 13-14*

isolating the concentrated L-amino acid from the medium or from the Escherichia cells, *page 1, lines 7-11; page 2, lines 13-25; page 3, lines 6-19*

wherein said modified microorganism comprises an inactivated poxB gene which prior to inactivation encodes a pyruvate oxidase, wherein inactivation is achieved by one or more methods of mutagenesis selected from the group consisting of deletion mutagenesis with deletion of at least one base pair in the poxB gene, insertional mutagenesis due to homologous recombination in the poxB gene, and transition or transversion mutagenesis with incorporation of a non-sense mutation in the poxB gene, -wherein the poxB gene prior to being inactivated comprises a polynucleotide sequence encoding a protein comprising SEQ ID NO:2. *page 1, lines 7-11; page 2, lines 13-25; page 3, lines 6-19; page 6, line 1 to page 8, line 15*

GROUND OF REJECTION TO BE REVIEWED ON APPEAL

1. The first ground of rejection to be reviewed on appeal is of claims 23, 25-26, 28, 33 and 42 under 35 USC 103(a) over Kikuchi (U.S. 5,932,453) in view of Shimizu (U.S. 5,445,948) and Chang (*J Bacteriol* 154:756-762, 1983) as identified in the Action at page 3.
2. The second ground of rejection to be reviewed on appeal is of Claim 27 under 35 USC 103(a) over Kikuchi in view Shimizu and Chang further in view of Matsui (U.S. 4,391,907) as identified in the Action at page 3.
3. The third ground of rejection to be reviewed on appeal is of Claims 39-41 under 35 USC 103(a) over Kikuchi in view Shimizu and Chang further in view of Dunican (U.S. 6,586,214).

There remains some obviousness-type double patenting rejections for which appellate review is not requested at this time.

ARGUMENT

The Examiner's reasoning of the rejection is as follows

Kikuchi teaches producing L-amino acids in *E. coli* but not the inactivation of the *poxB* gene as in the claims. Shimizu teaches that acetate has a negative effect on cell growth and Chang teaches that *poxB* catalyzes acetate from pyruvate. Therefore, it would have been obvious to inactivate the *poxB* gene to minimize the production of acetate thereby increasing the production of L-amino acids.

Appellants respectfully disagree with the findings of fact and conclusion based thereon that give rise to each of the rejections.

Shimizu discloses that the cell growth inhibiting substances can be removed by controlling the acetate concentration so as to be at most 17g/l (Col. 4, lines 44-50). Therefore a concentration above 17g/l acetate inhibits the growth of *E. Coli* bacteria. So it is not true that Shimizu teaches generally that acetate inhibits growth of *E. coli* in culture, as indicated in the rejection.

Chang, however, discloses that the feeble growth of modified bacteria is the result of the modification by inactivation of the pyruvate dehydrogenase complex by deletion of *aceFE* genes, so that the cells use the (energy consuming) pathway using the pyruvat oxidase complex (*poxB*). According to Chang this results in a worse growth with acetate, because these mutations lead to cells which are only able to grow with the acetate which is generated with *poxB* (page 757, right col. bottom).

This phenomenon has nothing to do with an inactivation of cell growth by acetate. So Chang in fact does not *teach* that *acetate inhibits growth of E. coli in culture* as the Examiner has generally stated in the Office Action cited above. Therefore there was no motivation for

those skilled in the art to combine Shimizu and Chang. Such a combination is a result of a hindsight, which is improper.

Chang further teaches that *it remains unclear why the growth of E. coli on the acetate produced by pyruvate oxidase is so feeble and that the role of these pathways in utilization of the acetate produced by pyruvate oxidase is under investigation* (page 762 last paragraph) which also indicates that the consequences of the genetic modifications were not predictable due to the complexity of biochemical regulation mechanisms.

Furthermore Chang does not use L-amino acid production strains but (modified) natural strains, which means that it cannot be concluded from alterations in natural strains which production relevant results might come out while making the same alterations in production strains.

The Examiner argues that because Chang teaches the role of *poxB* in producing acetate, inactivating *poxB* blocks acetate production and Shimizu teaches that acetate inhibits bacterial growth at some concentrations, one would have inactivated *poxB* in Kikuchi or Matsui's bacteria leading to a reasonable predictability of successfully producing a strain that would produce L-amino acids. This is erroneous.

Indeed, the pathway in which *poxB* is involved is not the primary source of acetate in a cell. As explained in Chang et al (1999) J. Bacteriol. 181 , 6657 (Results):

Introduction of a pta mutation did not completely eliminate acetate production during the growth phase. The acetate yield of the pta mutant on glucose, 0.11 mM z (g [dry weight])²¹, was reduced to one-fourth that of the wild type, 0.45 mM z (g [dry weight])²¹. Pyruvate oxidase (PoxB) presents a possible route for the generation of acetate in the pta mutant (Chang, Y.-Y., A.-Y. Wang, and J. E. Cronan, Jr. 1994. Expression of Escherichia coli pyruvate oxidase (PoxB) depends on the sigma factor encoded by the rpoS (katF) gene. Mol. Microbiol. 11:1019–1028.), because acetyl phosphate may be converted to

acetate and inorganic phosphate through the action of either AckA or nonenzymatic degradation (Brown, T. D. K., M. C. Jones-Mortimer, and H. L. Kornberg. 1977. The enzymatic interconversions of acetate and acetyl-coenzyme A in Escherichia coli. J. Gen. Microbiol. 102:327–336.). However, a further mutation in the poxB gene did not result in the complete elimination of acetate (Shin, S., and J. G. Pan. unpublished data.).

Therefore, it becomes clear that the pathway involving poxB is not the primary source for acetate. Many other sources can be found for amino acid or fatty acid metabolism as shown in the overview of the KEGG-Pathways.

It was generally known that acetate may significantly lower yield and productivity in different processes involving microorganisms. 9 g/l of acetate cause inhibition of cell growth (Xu et al., Applied Microbiology and Biotechnology, Volume 51, Number 5 / May 1999, page 564-571). This is not a particular problem of L-amino acid production.

Therefore, one who wanted to solve the problem of increasing the yield in fermentation processes for making L-threonine, L-valine or L-lysine would not have specifically sought to lower acetate formation and certainly not the inactivation of poxB. Therefore, contrary to the conclusion in the Action, which is clearly based on hindsight, the combination of art does not lead one to inactivate poxB in Kikuchi bacteria leading to a reasonable predictability of successfully producing a strain that would produce the L-amino acids as defined in the claims.

Accordingly, the rejections applied are not tenable.

The second and third grounds of rejection citing the same three references in view of Matsui or Dunican are for aspects of the certain dependent claims and are believed to be overcome for the same reasons that the rejection applying Kikuchi, Shimizu and Chang should be overcome. Therefore, Appellants do not separately argue these rejections.

Application No. 10/076,416
Appeal Brief

For the reasons stated in this Brief, Appellants respectfully request that the Examiner's rejections be withdrawn with direction to allow all of the claims pending in this application and pass this case to issue.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



Daniel J. Pereira
Attorney of Record
Registration No. 45,518

Customer Number

22850

Tel: (703) 413-3000
Fax: (703) 413 -2220
(OSMMN 08/07)

APPENDIX 1 (CLAIMS)

23. (Rejected) A process for fermentatively preparing an L-amino acid selected from the group consisting of L-threonine, L-valine, and L-lysine, comprising

fermenting a modified microorganism of the genus *Escherichia* which already produces L-amino acids before being modified for a time and under conditions suitable for the production of the L-amino acid;

concentrating the L-amino acid in the medium or in the *Escherichia* cells;

determining the concentration of the L-amino acids produced; and

isolating the concentrated L-amino acid from the medium or from the *Escherichia* cells,

wherein said modified microorganism comprises an inactivated *poxB* gene which prior to inactivation encodes a pyruvate oxidase, wherein inactivation is achieved by one or more methods of mutagenesis selected from the group consisting of deletion mutagenesis with deletion of at least one base pair in the *poxB* gene, insertional mutagenesis due to homologous recombination in the *poxB* gene, and transition or transversion mutagenesis with incorporation of a non-sense mutation in the *poxB* gene, wherein the *poxB* gene prior to being inactivated comprises a polynucleotide sequence encoding a protein comprising SEQ ID NO:2.

26. (Rejected) The process of Claim 23, wherein said L-amino acid is L-threonine.

27. (Rejected) The process of Claim 23, wherein said L-amino acid is L-valine.

28. (Rejected) The process of Claim 23, wherein said L-amino acid is L-lysine.

33. (Rejected) The process of Claim 23, wherein the modified microorganism is *Escherichia coli*.

39. (Rejected) The process of Claim 23, wherein inactivation is achieved by deletion mutagenesis with deletion of at least one base pair in the *poxB* gene.

40. (Rejected) The process of Claim 23, wherein inactivation is achieved by insertional mutagenesis due to homologous recombination.

41. (Rejected) The process of Claim 23, wherein inactivation is achieved by transition or transversion mutagenesis with incorporation of a non-sense mutation in the *poxB* gene.

42. (Rejected) The process of Claim 23, wherein the *poxB* gene prior to being inactivated comprises SEQ ID NO:1.

APPENDIX II (EVIDENCE)

1. The present specification, referenced in the arguments presented in this brief.
2. Chang et al (1999) J. Bacteriol. 181 , 6657 referenced in the paper filed on July 20, 2010.
3. An overview of the KEGG-Pathways referenced in the paper filed on July 20, 2010.
4. Xu et al., Applied Microbiology and Biotechnology, Volume 51, Number 5 / May 1999, page 564-571 referenced in the paper filed.

APPENDIX III (RELATED APPEALS AND INTERFERENCES)

None.

Acetate Metabolism in a *pta* Mutant of *Escherichia coli* W3110: Importance of Maintaining Acetyl Coenzyme A Flux for Growth and Survival

DONG-EUN CHANG,^{1,2} SOOAN SHIN,^{1†} JOON-SHICK RHEE,² AND JAE-GU PAN^{1*}

Bioprocess Engineering Division, Korea Research Institute of Bioscience and Biotechnology, Yusong, Taejeon 305-600,¹
and Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Yusong,
Taejeon 305-701,² Korea

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In order to study the physiological role of acetate metabolism in *Escherichia coli*, the growth characteristics of an *E. coli* W3110 *pta* mutant defective in phosphotransacetylase, the first enzyme of the acetate pathway, were investigated. The *pta* mutant grown on glucose minimal medium excreted unusual by-products such as pyruvate, D-lactate, and L-glutamate instead of acetate. In an analysis of the sequential consumption of amino acids by the *pta* mutant growing in tryptone broth (TB), a brief lag between the consumption of amino acids normally consumed was observed, but no such lag occurred for the wild-type strain. The *pta* mutant was found to grow slowly on glucose, TB, or pyruvate, but it grew normally on glycerol or succinate. The defective growth and starvation survival of the *pta* mutant were restored by the introduction of poly- β -hydroxybutyrate (PHB) synthesis genes (*phbCAB*) from *Alcaligenes eutrophus*, indicating that the growth defect of the *pta* mutant was due to a perturbation of acetyl coenzyme A (CoA) flux. By the stoichiometric analysis of the metabolic fluxes of the central metabolism, it was found that the amount of pyruvate generated from glucose transport by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) exceeded the required amount of precursor metabolites downstream of pyruvate for biomass synthesis. These results suggest that *E. coli* excretes acetate due to the pyruvate flux from PTS and that any method which alleviates the oversupply of acetyl CoA would restore normal growth to the *pta* mutant.

Escherichia coli excretes acetate as a major by-product of its aerobic metabolism (3). Acetate is produced from acetyl coenzyme A (CoA) via acetyl phosphate by the Pta-AckA (Pta, phosphotransacetylase; AckA, acetate kinase) pathway, which is reversible and constitutively expressed (6). The excreted acetate is reused via acetyl CoA synthetase (ACS) or the Pta-AckA pathway after the depletion of favored carbon sources such as glucose. ACS or the Pta-AckA pathway, showing higher or lower affinities for the acetate, respectively, activates acetate to acetyl CoA (24).

Aerobic acetate production depends on the growth rate. When the growth was limited by restricting the feeding rate of the carbon source, acetate excretion diminished and, under a critical growth rate (μ_c), was stopped in chemostat cultures of *E. coli* and *Aerobacter aerogenes* (13, 36, 42). The specific oxygen uptake rates increased linearly with increased growth rate under μ_c in both strains (36, 42). At growth rates higher than μ_c , specific oxygen uptake rates did not increase further and acetate production started in *E. coli* (36). Similar findings for batch cultures with various carbon sources that supported different growth rates were reported (3). Based on the effect of the relationship between acetate production and specific oxygen uptake rate upon growth rates and the fact that acetate biosynthesis is accompanied by equimolar substrate-level ATP generation, acetate excretion is regarded as an overflow me-

tabolism, which provides additional energy when the respiration capacity is saturated (3).

In the context of metabolic flux analysis, aerobic acetate production has been understood as an effect caused by the imbalance between the uptake of the substrate and the demand for biosynthesis and energy production (18). From stoichiometric flux analysis of the central metabolic pathways in *E. coli*, Holms proposed that the ratio of flux to energy supply does not limit growth and that consequently acetate excretion functions as a safety valve to balance excessive inputs to the fluxes of precursor metabolites for biomass synthesis (19). The fact that *E. coli* grown on glycerol or fructose does not excrete acetate was explained to be an effect of the restricted uptake of substrates. The maximal uptake rate of these substrates was not supposed to reach the threshold required to trigger acetate excretion (19). Though the analysis adequately demonstrated the existence of an imbalance between substrate uptake and the demands for anabolism, the exact cause of such an imbalance has not been explained.

A possible role of acetate metabolism is for the production of acetyl phosphate, which was recently found to be a phosphate donor for many signal transduction response regulators including CheY, PhoB, NR1, and OmpR (14, 27, 28, 33). It has been shown that the intracellular level of acetyl phosphate varies over a wide range, depending on the presence of a *pta* or *ackA* mutation, the phase of growth, and temperature (27, 35). Moreover, the *pta* mutant has been reported to be impaired in its ability to survive during glucose starvation, while the ability of the *ackA* mutant to survive remained the same as that of the parent strain (30). Therefore, providing an appropriate level of acetyl phosphate may be an important function of the acetate pathway in *E. coli*.

In this work, the growth phenotypes of a *pta* mutant were

* Corresponding author. Mailing address: Bioprocess Engineering Division, Korea Research Institute of Bioscience and Biotechnology (KRIBB), P.O. Box 115, Yusong, Taejeon 305-600, Korea. Phone: 82-42-860-4483. Fax: 82-42-860-4594. E-mail: jgpan@kribb4680.kribb.re.kr.

† Present address: Center for Vaccine Development, School of Medicine, University of Maryland, Baltimore, MD 21201.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source (reference)
<i>E. coli</i> strains		
W3110	F ⁻ IN(<i>rrnD-rrnE</i>)1	B. Bachmann
RR1	<i>supE44 hsdS20</i> (τ_B^- m_B^-) <i>ara-14 proA2 lacY1 galk2 rpsL20 xyl-5 mtl-1</i>	Laboratory collection
CP993	OW1 Φ (<i>pta-1::Tn10-lacZ</i>)	C. Park (35)
JP202	RR1 Φ (<i>pta-1::Tn10-lacZ</i>)	This work
JP231	W3110 Φ (<i>pta-1::Tn10-lacZ</i>)	This work
Plasmids		
pSK2665	<i>phbCAB</i> genes from <i>A. eutrophus</i>	A. Steinbüchel (34)
pKTPHB	pKT230 with the 5.2-kb <i>Bam</i> HI- <i>Eco</i> RI fragment bearing <i>phbCAB</i> genes from pSK2665	This work

investigated to understand the physiological roles of acetate metabolism. Several unexpected growth characteristics, such as a large decrease of the growth rate and the excretion of pyruvate and D-lactate, were found during previous investigations of *pta* mutants (12, 13, 21). Because the altered acetate metabolism must cause severe metabolic flux reorientation, which should result in the accumulation of by-products, the profiles of by-product accumulation, the consumption pattern of amino acids in complex medium, and the growth rates in cultures with a variety of carbon sources were examined in greater detail. Metabolic flux analysis, based on the calculated amounts of precursor metabolites required for the synthesis of biomass, revealed that pyruvate is always excessively generated because of the characteristics of the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). Therefore, it is proposed that acetate production in *E. coli* is an unavoidable consequence of substrate transportation via PTS and that the growth defect of the *pta* mutant is due to the unbalanced flux of acetyl CoA. Because the introduction of *phbCAB* genes redirecting acetyl CoA flux to poly- β -hydroxybutyrate (PHB) was found to improve the growth of the *pta* mutant, any method of alleviating the perturbed acetyl CoA flux would be expected to restore the normal growth of the *pta* mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. To construct *pta* mutants, a *pta-1::Tn10-lacZ* fusion was introduced into W3110 or RR1 by P1 transduction with lysates of CP993 (39). Plasmid pSK2665 harboring *phbCAB* genes from *Alcaligenes eutrophus* was provided by A. Steinbüchel (38). Plasmid pKTPHB was derived from pKT230 with the 5.2-kb *Bam*HI-*Eco*RI fragment containing *phbCAB* genes from pSK2665.

Culture media and growth conditions. Physiological characterizations were carried out by using M9 minimal medium (containing 6 g of Na₂HPO₄, 3 g of KH₂PO₄, 1 g of NH₄Cl, and 0.5 g of NaCl liter⁻¹ with 2 g of each carbon source liter⁻¹, tryptone broth (TB; containing 10 g of tryptone liter⁻¹ and M9 minimal salts), or Luria-Bertani broth (LB; 5 g of yeast extract, 10 g of tryptone, and 10 g of NaCl liter⁻¹). For the comparison of growth rates, amino acid consumption, and starvation survival, strains were cultivated at 37°C in 500-ml baffled flasks shaken at 200 rpm. Batch cultivations were carried out at 37°C in a 2.5-liter fermentor (Korea Fermentor Co., Inchon, Korea) which contained 1 liter of M9 minimal medium with 2 g of glucose liter⁻¹. The air flow rate was fixed at 1 liter min⁻¹, and dissolved oxygen was maintained above 20% of its saturation level by manually adjusting the agitation speeds in the range of 500 to 900 rpm. pH was kept constant at 7.0 by the addition of 2 M NaOH. Tetracycline (13 μ g ml⁻¹) or kanamycin (50 μ g ml⁻¹) was added as required. All the cultivations were repeated at least twice.

Analytical methods. Optical density (OD) was measured at 600 nm (Ultrospec 2000 UV/visible spectrophotometer; Pharmacia Biotech Ltd, Uppsala, Sweden), and dry cell weight was determined gravimetrically after the culture broth was centrifuged at 6,000 \times g, washed with distilled water, and dried overnight at 105°C. One OD unit was found to be equivalent to 0.39 \pm 0.05 g (dry cell weight) \cdot liter⁻¹. The glucose concentration in the medium was measured with a glucose analyzer (model 2300; YSI Co., Yellow Springs, Ohio). The concentrations of acetate, D-lactate, and pyruvate in the sample were assayed with enzymatic test kits (Boehringer, Mannheim, Germany). Concentrations of amino acids in the samples were determined with an amino acid analyzer (AminoQuant

1090; Hewlett-Packard, Avondale, Penn.). The synthesis of by-products was monitored by performing in vivo nuclear magnetic resonance (NMR) scans of whole cultures as described by Alam et al. (1, 26). Proton NMR spectra of culture broth samples were obtained with a Varian UNITY spectrometer operating at 500 MHz (Korea Basic Science Institute, Taejeon, Korea). The water peak was suppressed, the field was locked onto the solvent D₂O, and the H₂O peak at 4.65 ppm was used as the internal reference. Dimethyl sulfone (100 mM) was used as an internal standard (3.12 ppm) for the quantification of the fermentation products. PHB levels were determined by gas chromatography (Hewlett-Packard) with benzoic acid as an internal standard (5).

RESULTS

The *E. coli* W3110 *pta* mutant accumulates pyruvate, D-lactate, and L-glutamate instead of acetate. The growth phenotypes of an *E. coli* W3100 *pta* mutant were compared with those of wild-type strain W3100 to study the physiological changes due to the *pta* mutation. In glucose minimal medium, the *pta* mutant JP231 grew more slowly than its parental strain, W3110. The maximum specific growth rates were 0.49 and 0.60 h⁻¹ for JP231 and W3110, respectively (Fig. 1). While the biomass yields of both strains on glucose were the same, 0.077 g (dry weight) \cdot mM⁻¹, the specific glucose consumption rate of the *pta* mutant, 6.06 mM \cdot (g [dry weight] \cdot h)⁻¹, differed from that of wild type, 7.87 mM \cdot (g [dry weight] \cdot h)⁻¹. Introduction of a *pta* mutation did not completely eliminate acetate production during the growth phase. The acetate yield of the *pta* mutant on glucose, 0.11 mM \cdot (g [dry weight])⁻¹, was reduced to one-fourth that of the wild type, 0.45 mM \cdot (g [dry weight])⁻¹. Pyruvate oxidase (PoxB) presents a possible route for the generation of acetate in the *pta* mutant (8), because acetyl phosphate may be converted to acetate and inorganic phosphate through the action of either AckA or nonenzymatic degradation (6). However, a further mutation in the *poxB* gene did not result in the complete elimination of acetate (40). As previously reported (12, 21), pyruvate and D-lactate were excreted during the exponential growth of JP231, while in its parental strain (W3110) the excretion of pyruvate and D-lactate was quantitatively insignificant. Once glucose was exhausted, the *pta* mutant reused the excreted acetate mainly through ACS (24), pyruvate via pyruvate dehydrogenase (16) or pyruvate oxidase (8), and D-lactate via the NAD-independent, membrane-bound D-lactate dehydrogenase (D-LDH) (23).

Because metabolic perturbation was demonstrated by the secretion of byproducts, the possibility of acetyl CoA being redirected in the tricarboxylic acid (TCA) cycle was examined. Acetyl CoA incorporated into the TCA cycle may be oxidized completely to CO₂ or excreted as by-products such as intermediates of the TCA cycle or amino acids formed from such intermediates. No significant amounts of organic acids synthesized from intermediates of the TCA cycle were detected by NMR analysis. However, amino acid analysis showed that

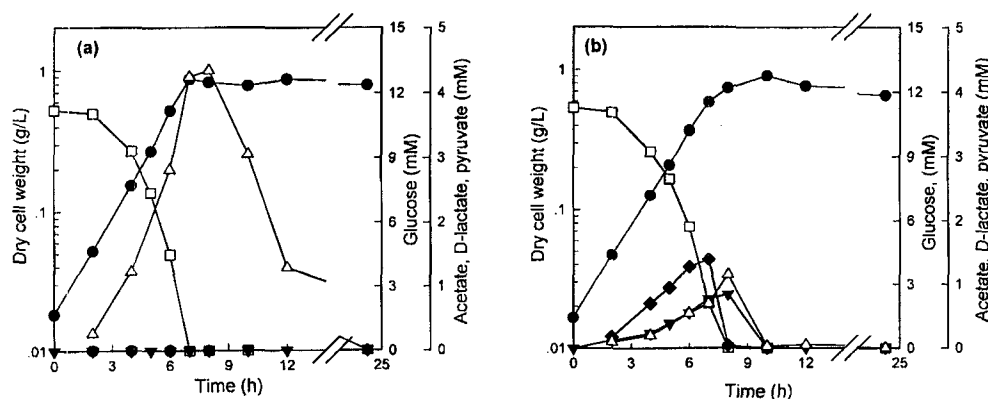


FIG. 1. Growth and by-product excretion profiles of W3110 (a) and JP231 (b). Strains were grown in an aerated fermentor containing M9 minimal medium supplemented with 2 g of glucose liter⁻¹. Symbols: ●, dry cell weight; □, glucose; △, acetate; ◆, pyruvate; ▼, D-lactate.

JP231 grown in glucose minimal medium accumulated glutamate, up to 0.33 mM from 12 mM glucose, while the amount produced by W3110 was insignificant (Fig. 2). No amino acids other than glutamate were detected. The sum of fluxes to pyruvate, D-lactate, and glutamate accounted for 96% of the acetate flux reduction in the *pta* mutant.

The *pta* mutant shows a different consumption profile of amino acids. The growth defect of the *pta* mutant on glucose and the accumulation of glutamate led us to check whether the consumption profiles of amino acids by the JP231 mutant, on TB containing M9 salts, differed from those of W3110. It is worth pointing out that the growth defect was more pronounced on TB containing M9 salts than on glucose minimal medium. On TB, the growth rate of JP231 was only 45% that of the wild-type strain, whereas it reached 78% of the control on glucose. Since amino acids serve as the primary carbon source, the levels of sequential consumption of amino acids by both W3110 and JP231 growing on TB containing M9 salts

were analyzed (Fig. 3). The consumption profiles of amino acids in the wild-type strain were consistent with those from a previous report (34). During the early exponential growth phase, L-serine was consumed exclusively; this was followed by the consumption of L-aspartate (Fig. 3a). In this phase, acetate accumulated and was later reused when consumption moved to L-glutamate, L-threonine, L-alanine, and glycine. In the stationary phase, L-arginine was the only amino acid to be consumed. Glycine was accumulated when the cells were consuming L-serine, L-threonine, and L-alanine, indicating an imbalance between the assimilation of such amino acids and their related catabolic pathways.

The *pta* mutant exhibited a different consumption pattern (Fig. 3b). Although its order of amino acid consumption was the same as that of W3110, there was a lag between the initial consumption of L-serine and other amino acids. After a brief lag, L-aspartate, L-threonine, L-alanine, glycine, and L-glutamate were assimilated. This retardation in the assimilation of amino acids is consistent with a previous report concerning the *nuo* mutant (34). Glycine accumulation was not observed, which reflected a decrease in the assimilation rate of such amino acids. As noted in the previous section, the concentration of L-glutamate increased before it was consumed. The accumulation level of acetate by the *pta* mutant was lower in TB containing M9 salts than in glucose minimal medium, which indicated that the assimilation of amino acids as the carbon source results in a lower flux to acetyl CoA than does that of glucose.

The *pta* mutant grows slowly on glucose, TB, or pyruvate but normally on glycerol or succinate. Maximum growth rates of JP231 on various carbon sources were compared with those of W3110, because the *pta* mutant had shown different growth defects in glucose and TB. JP231 grew more slowly than W3110 not only in glucose and TB containing M9 salts but also on fructose and pyruvate (Table 2) (17). The specific growth rates of the *pta* mutant on fructose and pyruvate were 75 and 45%, respectively, of those of the wild-type strain. However, on glycerol or succinate, the growth rates of the *pta* mutant were comparable to those of the wild type. Metabolism of substrates on which the *pta* mutant showed the growth defect is directly linked to pyruvate either by PTS (glucose and fructose), catabolism of amino acids (TB), or by pyruvate itself. Because PTS using PEP as the phosphate donor links the uptake of glucose or fructose with the stoichiometric coproduction of pyruvate, oversupplied pyruvate should be excreted as acetate. Therefore, the fluxes of pyruvate and consequently acetyl CoA

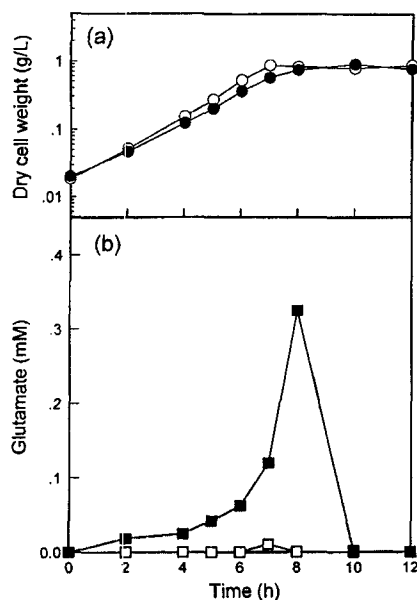


FIG. 2. Glutamate accumulation in a *pta* mutant grown on M9 glucose minimal medium. Profiles of growth (a) and glutamate accumulation (b) of W3110 (open symbols) and JP231 (solid symbols) are represented.

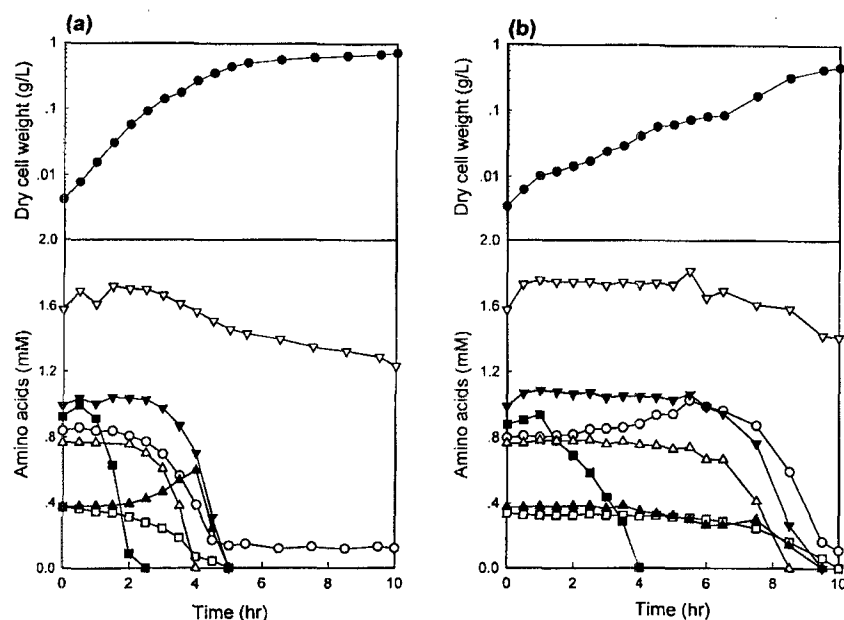


FIG. 3. Profiles of amino acid consumption patterns in W3110 (a) and JP231 (b) grown on TB. Symbols: ●, OD at 600 nm; ■, L-serine; □, L-aspartate; ○, L-glutamate; ▼, L-alanine; ▲, glycine; △, L-threonine; ▽, L-arginine.

in the *pta* mutant grown on PTS carbon sources would be expected to be severely perturbed, as is manifested by the excretion of pyruvate, D-lactate, and L-glutamate. In addition to the excretion of unusual by-products, the perturbation of acetyl CoA and possibly pyruvate fluxes in the *pta* mutant, in some way, causes the decrease in the growth rate.

Defective growth of the *pta* mutant is recovered by *phbCAB* introduction. In an attempt to check whether acetyl CoA flux is responsible for the decreased growth rate of the *pta* mutant, PHB synthesis genes (*phbCAB*) from *A. eutrophus* (38) were introduced into W3110 and JP231. Initially, the effects of introducing PHB synthesis pathway genes upon the distribution of acetyl CoA flux were examined by analyzing the by-products. In the wild-type strain, the introduction of *phbCAB* genes had no effect on the by-product pattern, in that acetate was excreted as the only product in M9 medium containing glucose (Fig. 4a). In contrast, the introduction of *phbCAB* genes repressed the excretion of the by-products, the characteristic of the *pta* mutant: excretion of pyruvate and D-lactate was diminished (Fig. 1b and 4b), and the maximum level of glutamate was lowered to one-sixth that associated with the *pta* mutant (data not shown). Moreover, the maximum level of acetate was also reduced to three-fourths that of the control mutant strain. The PHB content in the *pta* mutant harboring *phbCAB* was approximately twice those of the respective control strains. On LB supplemented with 20 g of glucose liter⁻¹, the PHB content of JP231 harboring pKT230 reached 24.5% of dry cell weight, while that of W3110 harboring the same plasmid was only 14.7%. This result indicates that the perturbed acetyl CoA flux was indeed redirected to PHB synthesis.

Since the perturbed acetyl CoA flux in the *pta* mutant can be directed to the synthesis of PHB, it might be expected that the introduction of PHB synthesis pathway genes would affect the maximum growth rate of the *pta* mutant. Recombinant strain JP231 with *phbCAB* was found to grow faster than a recombinant strain harboring pKT230, a control vector, on either TB or TB supplemented with 2 g of glucose liter⁻¹ (Table 3). In comparison, the recombinant strain W3110 harboring either

phbCAB or its vector grew slightly slower than the vector-free control strain. These results showed that the defective growth of the *pta* mutant is recovered, at least in part, by redirecting the acetyl CoA flux to PHB synthesis. In order to check whether this improved growth is strain specific, a comparison was made between the RR1 strain and its *pta* mutant (JP202), both harboring either plasmid pKT230 or pSK2665, a high-copy-number plasmids containing *phbCAB* (38). Recovery from the growth defect was more pronounced in JP202 (RR1 *pta* mutant) with pSK2665, showing a 1.2-fold-higher growth rate than the control strain on LB, while the growth rate of JP202 harboring pTKPHB remained unchanged. The growth rates of RR1 strains harboring either plasmid pKTPHB or pSK2665 decreased to 90% of that of the control strain (data not shown).

Starvation survival defect of the *pta* mutant is also recovered by the introduction of *phbCAB*. One of the most prominent phenotypes of the *pta* mutant is its poor starvation survival, which was proposed to be a result of its inability to provide acetyl phosphate (30). Encouraged by the finding that the introduction of *phbCAB* genes repressed the defective growth of the *pta* mutant, we examined the effect of the introduction

TABLE 2. Specific growth rates of *E. coli* W3110 and its *pta* mutant (JP231) on various carbon sources^a

Carbon source	Specific growth rate (h ⁻¹) of:	
	W3110	JP231
TB	1.30 ± 0.05	0.59 ± 0.02
Glucose	0.59 ± 0.05	0.49 ± 0.03
Fructose	0.44 ± 0.02	0.33 ± 0.05
Pyruvate	0.38 ± 0.03	0.17 ± 0.02
Glycerol	0.41 ± 0.01	0.42 ± 0.01
Succinate	0.31 ± 0.03	0.30 ± 0.04

^a The culture medium contained M9 salts with 2 g of each carbon source liter⁻¹. Results are the means of three independent experiments.

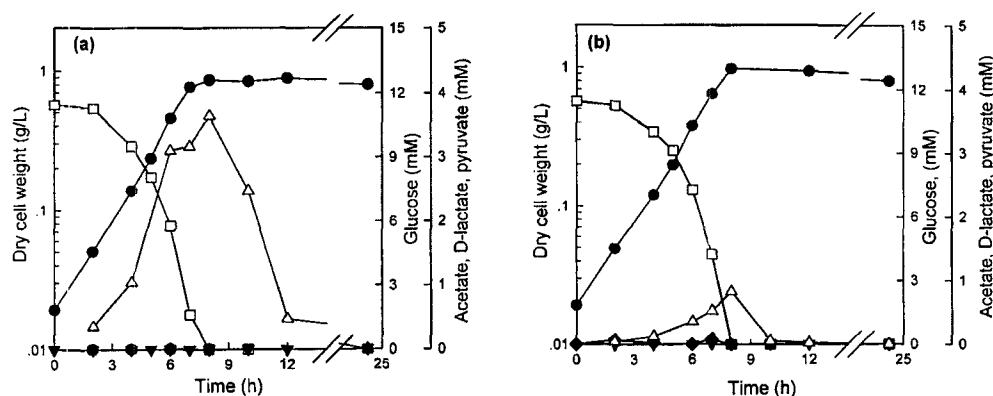


FIG. 4. Growth and byproduct excretion profiles of recombinant strain W3110 (a) and JP231 (b) harboring *phbCAB* genes from *A. eutrophus*. Strains were grown in an aerated fermentor containing M9 minimal medium supplemented with 2 g of glucose per liter. Symbols: ●, dry cell weight; □, glucose; △, acetate; ◆, pyruvate; ▼, D-lactate.

of *phbCAB* genes on the starvation survival of the *pta* mutant. W3110, JGP231, and their recombinant strains harboring pKTPHB were cultivated in M9 minimal medium with 0.2 g of glucose liter⁻¹. After the depletion of glucose, incubation was continued for 9 days and viable cells were counted as colonies plated on LB plates after appropriate dilution (30). As shown in Fig. 5, the viability of JP231 harboring *phbCAB* genes was indistinguishable from that of the wild-type strain while the *pta* mutant showed poor survival. This result indicates that the defective starvation survival of the *pta* mutant could also be recovered by redirecting acetyl CoA flux to PHB synthesis, demonstrating that managing the acetyl CoA flux rather than providing acetyl phosphate is important for the survival of *E. coli* under starvation conditions.

DISCUSSION

In this study, we showed that the *pta* mutation resulted in a growth defect and the excretion of unusual by-products. In order to illustrate further the metabolic basis of phenotypic changes in the *pta* mutant, the stoichiometric method of flux analysis by Holms (19) was adopted with a slight modification in that the TCA cycle does not function in the cyclic mode. Components of the TCA cycle function almost exclusively to provide three precursor metabolites, namely, oxaloacetate (OAA), α -ketoglutarate, and succinyl CoA (29). The TCA cycle does not function as an energy-generating cycle. Moreover, in a glucose medium, the expressions of the enzymes in the TCA cycle are repressed, and, especially, α -ketoglutarate dehydrogenase activity was absent (2, 31, 41). In Fig. 6a, which

shows a flux distribution of the precursor metabolites required for the synthesis of 1 g of biomass, the flux to PEP is insufficient and the insufficiency is resolved hypothetically by postulating a reverse flux through PEP synthetase, indicating the oversupply of pyruvate. A more likely way of supplying sufficient PEP flux is the uptake of additional glucose and its conversion to acetate, which is illustrated in Fig. 6b. In this case, it was assumed that 15% of the input glucose was excreted as acetate, reflecting the typical acetate yield of *E. coli* cultured on glucose. Now no hypothetical reverse flux from pyruvate to PEP is required. However, glucose transportation via PTS and the action of pyruvate kinase generate pyruvate (12.864 mmol) in excess of the required amount for precursor metabolites downstream of pyruvate, namely, acetyl CoA, α -ketoglutarate, and pyruvate (7.659 mmol). Oversupplied pyruvate and consequently acetyl CoA cannot be oxidized completely to CO₂ by the TCA cycle due to the repression of the TCA cycle enzymes, particularly of α -ketoglutarate dehydrogenase (2, 31, 41). Therefore, the pyruvate flux should be balanced by opting for acetate excretion (12). In the *pta* mutant, acetyl CoA flux is perturbed in

TABLE 3. Partial recovery of growth defect of *E. coli* W3110 *pta* mutant (JP231) by introduction of *phbCAB* genes from *A. eutrophus*^a

Strain	Specific growth rate (h ⁻¹) in:	
	TB	TB + glucose
W3110(pKT230)	1.29 ± 0.01	1.41 ± 0.03
W3110(pKTPHB)	1.09 ± 0.09	1.15 ± 0.05
JP231(pKT230)	0.62 ± 0.01	0.66 ± 0.01
JP231(pKTPHB)	0.67 ± 0.03	0.85 ± 0.05

^a The recombinant strains were grown on TB or TB supplemented with 2 g of glucose liter⁻¹. Results are the means of three independent experiments.

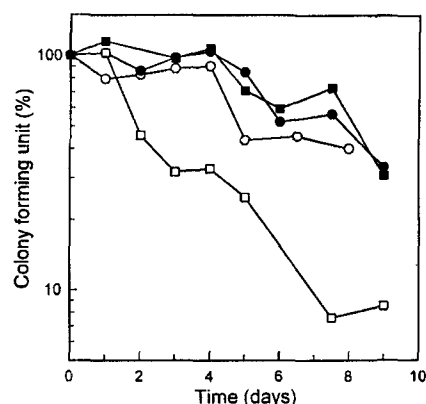


FIG. 5. Starvation survival of W3110, JP231, and their recombinant strains harboring *phbCAB* genes from *A. eutrophus*. Strains were grown in shaking flasks containing M9 minimal medium supplemented with 0.2 g of glucose liter⁻¹. After growth was arrested, incubation was continued for 9 days under the same condition. Viable cells were counted as colonies in LB plates after appropriate dilutions. One hundred percent viability corresponds to the number of viable cells counted 1 h after entering starvation. Symbols: ○, W3110; ●, W3110(pKTPHB); □, JP231; ■, JP231(pKTPHB).

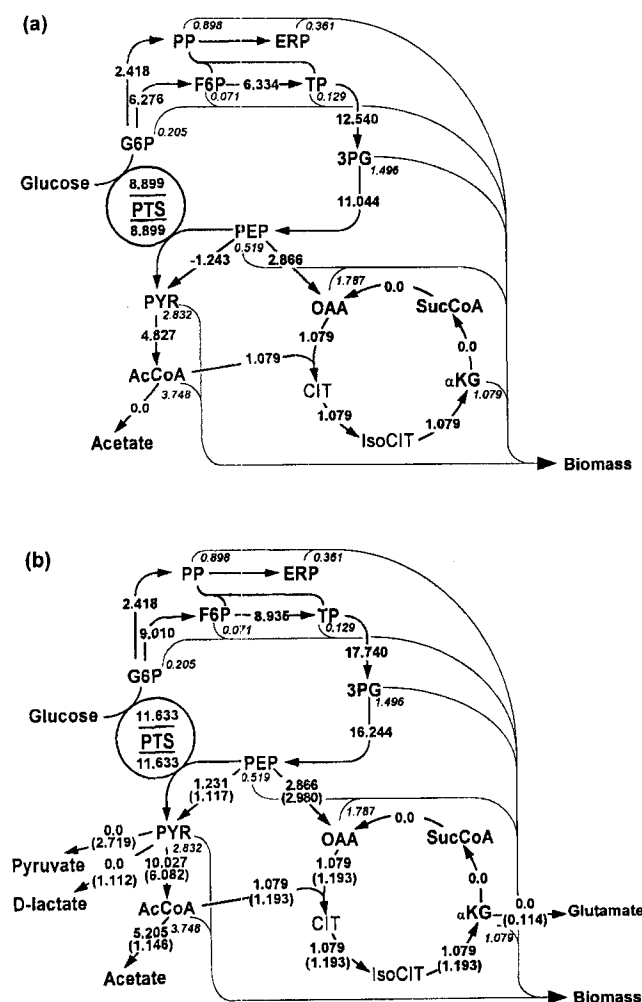


FIG. 6. Stoichiometric calculation of metabolic flux distribution in *E. coli*. The flux distribution was analyzed by Holms' method (16) with a slight modification in that the TCA cycle was assumed not to work in a cyclic mode but to function only for the generation of OAA, and α -ketoglutarate (2, 27, 36). Numbers in the middle of arrows indicate the fluxes through the specific metabolic step in the direction of the arrow. Italic numbers under precursor metabolites indicate the fluxes incorporated into biomass. A negative value means that the flux is in the direction opposite to the arrow. (a) Flux distribution in an ideal *E. coli* cell in which the central metabolism is working only for generating the exact amount of the required precursor metabolites and NADPH to make 1 g of biomass (25). The flux through a specific metabolic step was calculated by adding the amounts of precursor metabolites downstream of the metabolic step to make 1 g of biomass. In the resulting flux distribution, the amount of NADPH produced (4.319 mmol) was much smaller than that required to make 1 g of biomass (18.225 mmol). The shortage was covered by the uptake of additional (1.159 mmol) glucose and metabolizing it through the pentose phosphate pathway. (b) Flux distribution in wild-type *E. coli* in which the central metabolism works to supply the required amounts of precursor metabolites, NADPH, and ATP for 1 g of biomass and the production of acetate, which corresponds to 15% of input carbon. Numbers in parentheses indicate the fluxes for the *pta* mutant. The fluxes were calculated by adding the fluxes presented in panel a and the additional flux needed to produce acetate (wild type) or acetate, pyruvate, D-lactate, and glutamate (*pta* mutant). Abbreviations: AcCoA, acetyl CoA; α -KG, α -ketoglutarate; CIT, citrate; ERP, erythrose 4-phosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; IsoCIT, isocitrate; 3PG, 3-phosphoglycerate; PP, pentose phosphate; PYR, pyruvate; TP, triose phosphate; SucCoA, succinyl CoA.

that no apparent pathway(s) is available for treating the acetate CoA flux normally secreted by the wild-type strain.

In response to this metabolic flux perturbation, the *pta* mutant was found to excrete pyruvate, D-lactate, and L-glutamate.

The flux values in parentheses in Fig. 6b illustrate the theoretical flux distribution in the *pta* mutant, showing that the reduced acetate flux was redirected to the excretion of pyruvate, D-lactate, and L-glutamate. Excretion of pyruvate is a well-known characteristic of a mutant with a defective Pta-AckA pathway (12, 21). The accumulated acetyl CoA inhibits the activity of the pyruvate dehydrogenase complex, which causes the accumulation of pyruvate. Excretion of D-lactate may be the result of an elevated expression of D-LDH as well as pyruvate accumulation. In this regard, it is notable that the expression of *ldhA*, encoding fermentative D-LDH, increased and became independent of the medium pH by the mutations in the *pta* gene (7). Another possible response of a *pta* mutant to solve the problem involves the redirection of the acetyl CoA flux into the TCA cycle. Increased acetyl CoA flux into the TCA cycle requires an equivalent increase of PEP flux to OAA. The increased carbon flux must be excreted as by-products such as intermediates of the TCA cycle or amino acids if the TCA cycle does not function in a cyclic mode. As expected, the *pta* mutant excreted glutamate in an aerobic cultivation on glucose minimal medium or TB. Atkinson and Ninfa's recent finding that higher activity of glutamine synthetase increased the flux from α -ketoglutarate to glutamate and resulted in a decreased level of acetyl phosphate (4) is consistent with glutamate accumulation in the *pta* mutant. The excretion of glutamate confirms that α -ketoglutarate dehydrogenase is repressed during aerobic growth on glucose or amino acids (2, 31, 41). The flux to pyruvate, D-lactate, and glutamate in the *pta* mutant was equivalent to the reduction in acetate flux compared to that for the wild-type strain (Fig. 1b and 6b).

Why does the *pta* mutant grow slower than the wild-type strain if flux balance is accomplished by excreting pyruvate, D-lactate, and glutamate instead of acetate? Since acetate metabolism has been proposed to function as a secondary energy-yielding pathway, when the TCA cycle or respiration capacity or both are saturated (3), reduced energy generation due to the *pta* mutation may be responsible for the growth defect. However, the amount of ATP generated by the *pta* mutant was calculated as $73.425 \text{ mM} \cdot \text{g (dry weight)}^{-1}$, which reached 90.5% of the total energy generated by the wild type ($81.125 \text{ mM} \cdot \text{g [dry weight]}^{-1}$). In this calculation, it was assumed that all NADH generated was converted to ATP and that the P/O ratio was 1.5. Moreover, at higher growth rates, sufficient energy (ATP) is available from the Embden-Meyerhof-Parnas (EMP) pathway to minimize the energy-producing role of the TCA cycle (22). Therefore, a reduction in ATP generation cannot explain the large growth defect of the *pta* mutant, although the possibility of its partial contribution cannot be excluded. It is more plausible that the retarded growth on several carbon sources is due to the perturbation in the fluxes of acetyl CoA and pyruvate, which in turn would affect the substrate transport rate. In the *pta* mutant grown on glucose, the accumulation of pyruvate will lower the PEP/pyruvate ratio, which results in a lowered glucose uptake rate (25, 32). Moreover, accumulated acetyl CoA activates PEP carboxylase (Ppc), a PEP-consuming enzyme, which will decrease available PEP for glucose transport (20). Glutamate excretion in the *pta* mutant reflects the increased flux through Ppc. The slow uptake of substrate was also observed in the cultivation of the *pta* mutant on TB, which is demonstrated by the lags between the assimilation of amino acids and the disappearance of glycine accumulation. The retardation of the amino acid assimilation of the *pta* mutant is comparable to that of *nuo* mutants; a *nuo* mutant, defective in membrane-bound NADH dehydrogenase I, consumed L-glutamate slowly and glycine, L-threonine, and L-alanine poorly at the entry into stationary phase (34). The

slow or poor consumption of the amino acids was proposed to be a result of a higher NADH/NAD⁺ ratio, inhibiting both TCA cycle enzymes and enzymes functioning in the assimilation of amino acids (34). The *pta* mutation would decrease substrate uptake, and this in turn would lower the rate of growth.

If the growth defect of the *pta* mutant is caused by an unbalanced flux of acetyl CoA, the introduction of any pathway that can convert acetyl CoA to cell components or other metabolic end products should restore the growth of the *pta* mutant. As expected, when *phbCAB*, the PHB synthesis genes from *A. eutrophus*, were introduced, the growth defect of the *pta* mutant was restored (Table 3 and Fig. 5). The PHB content in the *pta* mutant was two times higher than that of the wild type, which demonstrated a redirection of the acetyl CoA flux normally destined for acetate synthesis. The difference between the PHB synthetic fluxes from acetyl CoA of the *pta* mutant (6.23 mmol) and the wild-type strain (3.31 mmol) was 2.92 mmol or 86% of the reduction of acetate flux (3.38 mmol), which shows that the increased flux directed to PHB synthesis actually contributes to the management of acetyl CoA flux. Diaz-Ricci et al. reported similarly that the expression of pyruvate decarboxylase and alcohol dehydrogenase genes from *Zymomonas mobilis* overcame the metabolic stress caused by the plasmid, enhancing growth and glucose uptake rates of a *pta* mutant to the observed values for the plasmid-free *pta* mutant (12). Particularly notable is that the starvation survival of the *pta* mutant was recovered by *phbCAB* introduction, which suggested that the poor survival of the *pta* mutant was due to problems of acetyl CoA flux rather than its inability to synthesize acetyl phosphate.

Our conclusion is that aerobic acetate production in *E. coli* is due to the oversupply of pyruvate from PTS, which strongly suggests that the production of acetate is a function of the balance of metabolic flux and not of the central metabolic rate or the growth rate itself. If the production of acetate is a matter of balance, why is acetate accumulation eliminated at lower growth rates? Two explanations are possible. First, the elimination of acetate excretion at lower growth rates may be due to the change in the glucose uptake system. Transport of substrate through the non-PTS system does not require a massive conversion of PEP to pyruvate, which may eliminate the pyruvate oversupply and in turn the excretion of acetate. In this context, it is worth noting that the non-PTS glucose uptake systems such as the maltose or galactose transport systems were found to be derepressed at lower growth rates in continuous culture (10, 11). Recently, Chen et al. showed that when *E. coli* exclusively transports glucose by the galactose transport system, a non-PTS Na symport system, the aerobic production of acetate was totally eliminated (9), which supports our contention that aerobic acetate production depends on the nature of the glucose uptake system. Second, the elimination of acetate excretion at lower growth rates may be the effect of derepression of TCA cycle enzymes, which permits a further metabolism of acetyl CoA. As TCA cycle enzymes are derepressed at lower growth rates in continuous culture, over-supplied pyruvate and acetyl CoA may be metabolized to CO₂ via the TCA cycle and acetate excretion may be eliminated. Further studies to examine the effects of changes of the glucose transport system or derepression of TCA cycle enzymes on acetate metabolism are in progress.

Another intriguing question arises here: why is the PTS chosen as the principal substrate uptake system if acetate excretion is due to PTS? Does PTS offer a competitive advantage in terms of outgrowth competition? Because the Mgl system coupled with LamB glycoporine appears to support slower

growth (15), it is tempting to suggest that the PTS system is used to achieve maximum growth rate at the expense of the growth yield due to acetate excretion. It is worth noting that a mutant with a higher glucose transport capacity evolved as one of the major populations during a long-term culture of *E. coli* in glucose-limited continuous culture (37). Furthermore, excreted acetate may be reused during the stationary growth phase when the principal substrates are exhausted (24), allowing cells to adapt to and survive starvation conditions.

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Pyruvate metabolism - Reference pathway (Enzyme)

[[Pathway menu](#) | [Pathway entry](#)]

Reference pathway (EC)	▼
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PYRUVATE METABOLISM

The diagram illustrates the central role of pyruvate in metabolism. Key features include:

- Central Hub:** Pyruvate is the central molecule, with numerous arrows indicating its conversion to and from other metabolites.
- Enzymes:** Specific enzymes are represented by boxes with codes, such as 1.1.1.21 for Lactate dehydrogenase, 2.7.1.40 for Pyruvate decarboxylase, and 1.1.1.39 for Oxaloacetate decarboxylase.
- Pathways:** Various metabolic pathways are highlighted in rounded rectangles, including Glycolysis, Glyoxylate metabolism, Citrate cycle, and Leucine biosynthesis.
- Interconnections:** The map shows how pyruvate is linked to other major metabolic pathways, such as the citrate cycle, gluconeogenesis, and amino acid metabolism.

